Cathepsin B but not Cathepsins L or S contributes to the pathogenesis of Unverricht-Lundborg progressive myoclonus epilepsy (EPM1)

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Abstract

The inherited epilepsy Unverricht-Lundborg disease (EPM1) is caused by loss of function mutations in the cysteine protease inhibitor, cystatin B. Since cystatin B inhibits a class of lysosomal cysteine proteases called cathepsins, we hypothesized that increased proteolysis by one or more of these cathepsins is likely to be responsible for the seizure, ataxia and neuronal apoptosis phenotypes characteristic of EPM1. To test this hypothesis and to identify which cysteine cathepsins contribute to EPM1, we have genetically removed three candidate cathepsins from cystatin B-deficient mice and tested for rescue of their EPM1 phenotypes. Whereas removal of cathepsins L or S from cystatin B-deficient mice did not ameliorate any aspect of the EPM1 phenotype, removal of cathepsin B resulted in a 36 to 89 percent reduction in the amount of cerebellar granule cell apoptosis depending on mouse age. The incidence of an incompletely penetrant eye phenotype was also reduced upon removal of cathepsin B. Since the apoptosis and eye phenotypes were not abolished completely and the ataxia and seizure phenotypes experienced by cystatin B-deficient animals were not diminished, this suggests that another molecule besides cathepsin B is also responsible for the pathogenesis, or that another molecule can partially compensate for cathepsin B function. These findings establish cathepsin B as a contributor to the apoptotic phenotype of cystatin B deficient mice and humans with EPM1. They also suggest that the identification of cathepsin B substrates may further reveal the molecular basis for EPM1.

Key Words: EPM1, Unverricht Lundborg, progressive myoclonus epilepsy, cystatin B, stefin B, cathepsin, apoptosis, cell death, ataxia, lysosome.

Introduction

Progressive myoclonus epilepsy of the Unverricht-Lundborg type (EPM1) is inherited in an autosomal recessive fashion and affects as many as one in 20,000 individuals. The symptoms of EPM1 typically begin between the ages of 8 and 13, and manifest as lightning-like myoclonic muscle jerks and tonic-clonic seizure attacks. EPM1 is progressive and results in mental deterioration and severe ataxia (Harriman and Miller, 1955; Koskiniemi, et al., 1974; Eldridge et al., 1983). Several years ago, our laboratory used a positional cloning strategy to discover that loss-of-function mutations in the cystatin B gene are responsible for this debilitating disease (Pennacchio et al., 1996). To help determine the molecular mechanisms of EPM1, we created cystatin B-deficient mice and found that they develop many of the same symptoms as children with EPM1, including a striking loss of cerebellar granule neurons by apoptotic cell death (Pennacchio et al., 1998; Shannon et al., 2002).

Cystatin B is a member of a large family of evolutionarily related cysteine protease inhibitors (Turk and Bode, 1991; Turk et al., 1997). Cystatin B appears to be diffusely distributed throughout the cytoplasm of all cell types (Calkins et al., 1998; Pennacchio and Myers, 1996), and is a strong, reversible inhibitor of most of the 11 currently known human cysteine cathepsins. Although these cathepsin proteases reside in the lysosome and mainly function to non-selectively catabolize proteins, many cathepsin family members also have essential tissue- and substrate-specific functions (Reviewed in Chapman et al., 1997; Turk et al., 2000). Recently, increasing evidence has indicated that cathepsins play an important role in the initiation and or propagation of apoptotic cell death in some contexts. Theoretically, the proteolytic actions of cathepsins

could participate in the apoptotic process at many levels. However, only two modes of cathepsin action have received experimental support to date. First, certain cathepsins may cleave and thereby directly activate caspases, the pro-apoptotic mediators of the apoptotic cascade (Schotte, et al., 1998; Vancompernolle et al., 1998; Ishisaka et al. 1999). Second, cathepsins can activate other members of the apoptotic cascade such as the pro-apoptotic Bcl-2 family member Bid (Stoka et al., 2000; Reiners et al., 2002). Thus, cathepsins have the potential to induce or propagate apoptosis, and by virtue of escaping from lysosomes and coming into contact with the cytoplasmic apoptotic machinery, may allow the cell to sense lysosomal damage.

Cathepsin activity was found to be significantly increased in lymphoblasts from EPM1 patients (Rinne et al., 2002). To establish whether the unregulated activity of cysteine cathepsins is responsible for the phenotypes characteristic of EPM1 and to identify which cathepsins are involved in this process, we chose to eliminate the likely candidate cathepsins B, L, and S individually from cystatin B-deficient EPM1 mice. We hypothesized that removing the culpable cathepsin would decrease apoptosis and alleviate the phenotypes of EPM1 disease mice. We found that the removal of cathepsin B, but not cathepsin S or L, from cystatin B-deficient mice greatly reduced the neuronal apoptotic phenotype caused by cystatin B deficiency. However, its removal did not reduce the severity of the EPM1 seizure or ataxia phenotypes, suggesting that neuronal apoptosis may not be as responsible for the phenotypes of ataxia and seizures as was previously thought. Nevertheless, cathepsin B likely plays a critical but non-exclusive role downstream of cystatin B in EPM1 pathogenesis.

Methods:

Mice: Cystatin B-deficient mice were created as described previously (Pennacchio et al., 1998). Cathepsin B (Deussing et al., 1998; Halangk et al., 2000) and L-deficient mice (Nakagawa et al., 1998; Roth et al., 2000) were provided by Dr. Christoph Peters. Cathepsin S-deficient mice (Nakagawa, et al., 1999; Shi et al., 1999) were a gift from Dr. William Brisette. Mice heterozygous for cystatin B were bred to cathepsin B, L or S heterozygous mice individually to create progeny that were heterozygous for both cystatin B and cathepsin deletions. These double heterozygotes were intercrossed to produce mice doubly-deficient for both cystatin B and each cathepsin in addition to all the necessary controls (cystatin B +/+ cathepsin +/+, cystatin B -/- cathepsin +/+, cystatin B +/+ cathepsin -/-). All mice were genotyped by PCR as previously described.

The severity of cystatin B-deficient phenotypes is dependent on mouse strain (Pennacchio et al., 1998). The pure 129Sv strain of cystatin B-deficient mice display prominent ataxia, seizure and apoptosis phenotypes, whereas cystatin B deficient mice on a C57BL/6 background have diminished phenotypes. For this reason, breeding cathepsin L-deficient mice (C57BL/6 background) to cystatin B-deficient mice resulted in less severe baseline phenotypes than did cathepsin B-deficient (129Sv / C57BL/6 mixed background). Breeding cystatin B-deficient mice to cathepsin S-deficient mice (DBA background) produced an intermediate level of phenotype severity.

Cathepsin transgenic mice were constructed as follows: human cathepsin B, L, H and S BAC clones were identified by sequence homology search or by PCR from a pooled BAC library

(CTD-2284F1, CTD-2062O4, RP11-268I9, and CTD-2326G15 respectively). BAC DNA was micro-injected into fertilized FVB blastocysts and several lines of founder transgenic mice were identified by PCR. Transgene overexpression was confirmed by comparing the quantitative PCR results of reverse transcribed cDNA from a variety of transgenic and wild type tissues, using primers that recognize both human and mouse cathepsin sequences.

TUNEL Analysis: Mice were transcardially perfused with 4% paraformaldehyde and brains were postfixed in 4% paraformaldehyde overnight before being paraffin embedded and sectioned. The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay for apoptosis was performed according to the manufacturer's instructions for paraffin embedded tissues (Cat. # G3250, Promega). The number of fluorescently labeled apoptotic cells was quantitated by using Zeiss KS 300 image analysis software. Three fields from each brain hemisphere were imaged for a total of six fields per mouse. For cathepsin B and L double knockout experiments the number of apoptotic cells was measured in two-month old mice (n= 4), four-month old (n= 4), and eight-month old mice (n= 9-11) of each genotype. For cathepsin S double knockout experiments the number of apoptotic cells was measured in two-month old mice (n= 6), four-month old (n= 3-5), and eight-month old mice (n= 3-4) of each genotype. An unpaired, two-tailed Student's T test was used to evaluate the differences between genotypes.

Ataxia Measurements: Two-month old mice (n=4), four-month old mice (n=4) and eight-month old mice (n=9-11) of each genotype were tested for ataxia by using a rotating-rod apparatus (Columbus Instruments) at both 0 rpm or 2 rpm speeds. Mice were placed on the rod

for a total of five consecutive trials of one minute duration each. Mice remaining on the rod for one minute received a perfect score. Unpaired, two-tailed Student's T test was used to evaluate the differences between genotypes.

A second ataxia measurement, the gait variability paradigm (Chakrabarti, et al. 1998; Rushton and Steinberg, 1963), was also used to test two-month old mice (n=4), four-month old mice (n=4) and eight-month old mice (n=9-11) of each genotype. After dipping the hind paws in ink, the mice were allowed to run from the entrance end of an enclosed 60 cm long runway lined with paper to the exit end. The variability of spacing between footsteps as marked by the ink is an index of ataxia. The mean log variance of the distance between hind paws for at least ten successive steps was calculated for each mouse and averaged for each of the two testing repetitions. An average mean log variance of greater than one is an indication of ataxia.

Cortical EEG and Seizure Measurements: Doubly-deficient cystatin B KO, cathepsin KO and singly-deficient cystatin B KO mice were observed at different ages for evidence of seizures. For electrocorticographic (EEG) seizure recordings, silver wire electrodes (0.005" diameter) soldered to a microminiature connector were implanted bilaterally into the subdural space over the frontal and parietal cortex of anesthetized mice several days prior to recording. Cortical activity and behavior were recorded using a digital video/electroencephalograph (Stellate Systems) from 8-9.5 month old mutants and controls moving freely in the test cage for prolonged periods (>2 hours) during a minimum of five sessions, including overnight recordings.

Results:

Production of cystatin B, cathepsin doubly-deficient mice: To determine whether any of the candidate cathepsins B, L or S contribute to the cystatin B null phenotypes, we constructed three lines of doubly-deficient mice: 1.) cystatin B / cathepsin L doubly-deficient mice, 2.) cystatin B / cathepsin S-doubly-deficient mice, and 3.) cystatin B / cathepsin B doubly-deficient mice. Cystatin B -/- cathepsin B -/- mice and cystatin B -/- cathepsin S -/- were phenotypically normal at birth and were born at the expected Mendelian ratio. As previously described for singly cathepsin L-deficient mice, the double deficient cystatin B -/- cathepsin L -/- mice had increased mortality and periodic hair loss (Roth et al., 2000).

Granule cell apoptosis in cystatin B, cathepsin doubly-deficient mice:

Cathepsin L does not contribute to apoptosis in the absence of cystatin B: We first tested cathepsin L for involvement in the apoptotic phenotype of EPM1 by measuring the number of TUNEL positive cerebellar granule cell cells at two, four and eight months of age. As expected, there was little to no visible granule cell apoptosis in cystatin B +/+ cathepsin L +/+ wild type mice at either two months (Figure 1A) or eight months of age (Figure 1D). At two months of age, cystatin B -/- cathepsin L +/+ disease mice displayed widespread granule cell apoptosis (average of 41 apoptotic granule cells per field) (Figure 1B) as did doubly-deficient cystatin B -/- cathepsin L -/- mice (31 apoptotic cells per field) (Figure 1C). This difference was not statistically significant (P= 0.416) (Figure 1S). At eight months of age cystatin B -/- cathepsin L +/+ mice had an average of 40 apoptotic granule cells per field (Figure 1E), whereas doubly-deficient cystatin B -/- cathepsin L -/- mice had 29 (Figure F). This small decrease in the amount

of apoptotic granule cell death was not statistically significant (P=0.095) (Figure 1S), suggesting that cathepsin L alone is not responsible for the apoptosis observed in this disease.

Cathepsin S does not contribute to apoptosis in the absence of cystatin B: We next tested cathepsin S for involvement in the apoptotic phenotype of EPM1. There were very few apoptotic granule cells visible in cystatin B +/+ cathepsin S +/+ wild type mouse brains at either two (Figure 1G) or eight months (Figure 1J) of age. Cystatin B-/- cathepsin S +/+ disease mice experienced abundant granule cell apoptosis at two months of age (Figure 1H) and less apoptosis at eight months of age (Figure 1K), as did doubly-deficient cystatin B -/- cathepsin S -/- mice (Figures 1I and 1L). Because there was no statistically significant reduction in the amount of granule cell apoptosis in the cystatin B-deficient disease mice upon removal of cathepsin S at two, four or eight months of age (P=0.199, P=0.741, P=0.870 respectively) (Figure 1T), this indicates that cathepsin S alone does not cause granule cell apoptosis in the absence of cystatin B.

Cathepsin B contributes to apoptosis in the absence of cystatin B: Finally, we tested cathepsin B for involvement in the apoptotic phenotype of EPM1. Almost no TUNEL-positive granule cells were present in cystatin B +/+ cathepsin B +/+ wild type mice at either two months (Figure 1M) or eight months (Figure 1P) of age. We observed extensive granule cell apoptosis in two-month old cystatin B -/- cathepsin B +/+ disease mice (average of 162 apoptotic cells per field) (Figure 1N) but much less apoptosis in doubly deficient cystatin B -/- cathepsin B -/- mice (average of 18 apoptotic cells) (Figure 1O). This represents an 89% reduction in the amount of apoptosis upon removal of cathepsin B (P = 0.006) (Figure 1U). A similar trend towards

decreased apoptosis was also present at four months of age when cystatin B -/- cathepsin B +/+ mice had an average of 203 apoptotic cells per field and cystatin B -/- cathepsin B -/- mice displayed 130 apoptotic cells per field. Due to cell count variability between mice of the same genotype, this 36% decrease did not reach statistical significance (P = 0.109) (Figure 1U). At eight months of age, the reduction in apoptosis was dramatic: cystatin B -/- cathepsin B +/+ mice had an average of 90 apoptotic cells per field (Figure Q), whereas cystatin B -/- cathepsin B -/- mice had only 35 apoptotic cells per field (Figure R) for a 61% reduction in apoptosis (P = 0.00002). This finding suggests that cathepsin B contributes to the initiation and/or propagation of apoptosis when it is not held in check by its physiological inhibitor, cystatin B. However, because the apoptosis was not completely abolished by removal of cathepsin B, it is likely that another cathepsin or unknown factor acts together with cathepsin B to achieve apoptosis. Alternatively, it is possible that another cathepsin causes apoptosis at a lower efficiency than does cathepsin B, and this activity is detectable only when cathepsin B is missing.

Although we present only terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining of granule cells as evidence for apoptosis, we have confirmed the presence of apoptosis by both Western blotting and immunocytochemistry of cerebellar tissues using activated caspase-3 antibodies (data not shown).

Ataxia measurements in cystatin B, cathepsin doubly-deficient mice: We have previously shown that aged cystatin B-deficient mice experience ataxia, consisting of poor balance while moving and a lack of motor coordination. To determine whether any of the three candidate cathepsins (B, L, S), contributes to this phenotype in the absence of cystatin B, we tested each of

the three doubly-deficient mice described above for reduced ataxia using a rotating rod apparatus and a gait variability test (Chakrabarti, et al. 1998).

Cathepsin L does not contribute to ataxia in the absence of cystatin B: Wild type cystatin B +/+ cathepsin L +/+ mice performed well on the rotorod ataxia test at all ages, remaining on the 0 rpm rod (Figure 2A) or 2 rpm rod (Figure 2B) for each full 60 second trial. Cystatin B -/- cathepsin L +/+ disease mice performed well at two and four months of age, but by eight months of age experienced ataxia as evidenced by a decreased ability to stay on either the stationary or rotating rod during the trial. The doubly-deficient cystatin B -/- cathepsin L -/- mice had nearly normal capability at two and four months of age, but by eight months of age were even less able to stay on the stationary or rotating rod than the cystatin B -/- cathepsin L +/+ mice. There was no statistically significant difference in rotorod ability at either 0 or 2 rpm between cystatin B -/- cathepsin L +/+ and cystatin B -/- cathepsin L -/- mice ($P=0.06$ and $P=0.68$ respectively). Similar findings were obtained with the gait variability test to measure ataxia in these mice (Figure 2C). Specifically, cystatin B -/- cathepsin L+/+ mice and cystatin B -/- cathepsin L -/- mice displayed ataxic symptoms only at eight months of age and the removal of cathepsin L from cystatin B-deficient mice did not reduce the severity of ataxia. Thus, cathepsin L does not appear to be responsible for the ataxia phenotype in cystatin B-deficient mice.

Cathepsin S does not contribute to ataxia in the absence of cystatin B: We also tested whether the removal of cathepsin S from cystatin B-/- mice resulted in changes in the severity of the ataxia phenotype. Due to quarantine housing procedures, we were able to use only the stride variability test to assess ataxia in these mice. As expected, cystatin B +/+ cathepsin S +/+ mice experienced no ataxia at any age, as evidenced by low stride variability scores (Figure 2D).

Cystatin B-/- cathepsin S +/+ mice exhibited a tendency towards more ataxia by eight months of age, as did cystatin B -/- cathepsin S-/-. The difference in ataxia between these singly and doubly-deficient mice was not statistically different, leading us to conclude that cathepsin S alone does not cause the ataxia phenotypes in the absence of cystatin B.

Cathepsin B does not contribute to ataxia in the absence of cystatin B: Finally, we tested cathepsin B for involvement in the ataxia phenotype. As expected, cystatin B +/+ cathepsin B +/+ wild type mice did not exhibit ataxia at any age tested, either with the rod at 0 rpm (Figure 2E) or 2 rpm (Figure 2F). Cystatin B -/- cathepsin B +/+ disease mice displayed nearly normal locomotor ability at two and four months of age, but by eight months performed poorly at both speeds. Removal of cathepsin B from cystatin B-deficient mice resulted in a worse performance on the stationary and rotating rod, instead of an improved one as had been predicted from the reduction of granule cell apoptosis observed in these mice. There was no statistically significant difference in rotorod ability at either 0 or 2 rpm between eight month old cystatin B -/- cathepsin B +/+ and cystatin B -/- cathepsin B -/- mice (P=0.76 and P=0.16 respectively). Upon further investigation, we found that the singly-deficient cathepsin B-deficient mice had an inherent ataxic phenotype evident by four months of age. This previously unknown mild phenotype of cathepsin B -/- mice appears to combine in an additive fashion with that of the cystatin B -/- mice to produce the severe ataxia characteristic of cystatin B -/- cathepsin B -/- mice. The second test for ataxia (Figure 2G) revealed the same trend, with cystatin B -/- cathepsin B -/- mice exhibiting slightly more variable stride lengths than the cystatin B-/- cathepsin B +/+ mice and the cystatin B +/+ cathepsin B -/- displaying more variability than wild type mice. Thus, cathepsin B is not likely to be responsible for the ataxia phenotype, despite its apparent contribution to the death of

granule cells. These data also suggest that the ataxia and granule cell apoptosis phenotypes are not necessarily linked in EPM1 disease.

Seizure measurements in cystatin B, cathepsin doubly-deficient mice: During sleep, cystatin B-deficient mice experience frequent seizures that consist of ear, vibrissae, head, tail and whole body twitches and jolts (Pennacchio et al., 1998). To determine whether the removal of any of the three cathepsins from cystatin B-deficient mice abolished the seizure phenotype, we first made visual observations of each double knock out line. Multiple doubly-deficient mice from all three lines experienced seizures upon falling asleep. To better characterize the seizure type and severity, we performed EEG recordings from doubly-deficient mice from each line and compared them to recordings from singly-deficient cystatin B -/- disease mice.

Similar to singly deficient cystatin B -/- mice (Figure 3A), the doubly deficient cystatin B -/- cathepsin L -/- (Figure 3B) and cystatin B -/- cathepsin B -/- mice (Figure 3C) displayed frequent spontaneous axial myoclonic jerks associated with a bilateral 150-300 msec electrographic discharge, typically at the rate of 10-15 hour (cystatin B -/- cathepsin S-/- could not be tested due to quarantine precautions). Myoclonus occurred both during wakefulness and sleep, and often the discharge was sufficient to awaken the animal. The baseline cortical activity of singly deficient (Figure 3D) and doubly deficient mice (Figure 3E) showed periods of normal, low amplitude desynchronized EEG, and at times, interictal discharges not associated with myoclonic jerks were present, as were bilateral electrographic seizure discharges. Mice showed no clonic or tonic movements during these seizures. Since the seizures and myoclonic jerks recorded from

the various genotypes were essentially identical regardless of cathepsin content, we conclude that cathepsins B, L and S alone do not contribute significantly to the seizure phenotype.

Eye phenotype in cystatin B, cathepsin doubly-deficient mice: In our initial characterization of the singly-deficient cystatin B mice on the 129 Sv background, we noted that approximately 35% of these mice experienced a mild eye phenotype consisting of corneal lesions and or serous exudate in one or both eyes (Pennacchio et al., 1998). In the present study, we quantitated the number of eight month old doubly-deficient mice with this eye phenotype to determine whether the removal the various cathepsins influenced the eye phenotype severity. Whereas two of ten (20%) cystatin B -/- cathepsin L +/+ mice had the eye phenotype, five of nine (56%) cystatin B -/- cathepsin L -/- mice had the eye phenotype. This result was surprising since we expected that removing cathepsins might reduce the severity of phenotypes, not exacerbate them. Interestingly, the cystatin B deficient eye phenotype is characterized in part by a loss of corneal epithelium and keratosis (Pennacchio et al., 1998), whereas mice deficient for cathepsin L have been shown to experience hyperkeratosis and epithelial cell abnormalities (Roth et al., 2000). Thus, the increased number cystatin B -/- cathepsin L -/- mice with the eye phenotype may be due to the already compromised epithelial cells of cathepsin L deficient mice.

We next determined that one of three cystatin B -/- cathepsin S +/+ mice had the eye phenotype (33%) whereas zero of four cystatin B -/- cathepsin S -/- had the phenotype. This apparent reduction in incidence of the eye phenotype upon removal of cathepsin S may be a real effect, but is not conclusive due to the small number of animals available for testing.

Finally, we counted three of eight cystatin B -/- cathepsin B +/+ mice (38%) and one of seven cystatin B -/- cathepsin B -/- mice (14%) with the eye phenotype. This reduced number of doubly deficient mice with eye lesions suggests that cathepsin B may be involved in the pathogenesis of this eye phenotype and corroborates the previously described finding of cathepsin B contribution to other EPM1 phenotypes.

Cathepsin overexpressor mice: As a complimentary approach to making cystatin B / cathepsin doubly deficient mice, we have also generated cystatin B-deficient / cathepsin overexpressing transgenic mice (Tg). We postulated that increasing the expression of candidate cathepsins B, L, H, or S would exacerbate the cystatin B -/- induced apoptosis, ataxia or seizure phenotypes and might thereby help identify which cathepsin is responsible for these different EPM1 phenotypes. To produce cystatin B deficient mice with only one copy of the cathepsin transgene, we bred cystatin B heterozygote / cathepsin Tg + mice to cystatin B heterozygote / cathepsin Tg – mice. Although our breeding scheme was predicted to yield the cystatin B -/- cathepsin Tg + genotype in 12.5 % of pups born, we recovered significantly less than this predicted ratio for cathepsin B and L transgenic lines, whereas the cathepsin H and S lines were produced at the expected frequency. Specifically, of 175 total pups born, we recovered only 3 pups (or 2 %) with the cystatin B -/- cathepsin B Tg + genotype. Similarly, we recovered only 1 of 122 pups (or >1 %) with the cystatin B -/- cathepsin L Tg + genotype. This reduced genotype frequency indicates that the overexpression of cathepsins B or L and simultaneous removal of their endogenous inhibitor cystatin B can be a lethal combination.

While the low number of mice generated in this experiment precludes us from drawing any definite conclusions, we did attempt to measure granule cell apoptosis and ataxia in the individuals that remained. Overexpressing cathepsin S or H in cystatin B-deficient mice did not result in a significant difference in the severity of apoptosis or ataxia (Data not shown). We observed a trend towards increased ataxia when cathepsin B was overexpressed in cystatin B-deficient mice (Data not shown). These findings lend support to the previously described double knockout data, showing that cathepsin B contributes to the pathogenesis of EPM1, whereas cathepsin S does not appear to.

Discussion

Humans and mice that lack the ubiquitously expressed cysteine protease inhibitor cystatin B experience seizures, ataxia and neuronal apoptosis, a disease known as progressive myoclonus epilepsy (EPM1). To determine the downstream molecular consequences of cystatin B deficiency, we attempted to identify which of the cathepsin proteases that cystatin B normally regulates is the cause of EPM1 phenotypes. To narrow the number of cathepsin candidates to test, we focused on well-characterized, abundant cysteine cathepsins that displayed detectable brain expression, and for which mouse deletion mutants had already been constructed (Uchiyama et al., 1994). We hypothesized that the lack of regulation of one cathepsin might cause all of the observed phenotypes, or, alternatively, that each cathepsin might be responsible for a portion of the different phenotypes. To test these possibilities, we removed cathepsins B, L, or S individually from cystatin B-deficient mice and assessed the degree of apoptosis, ataxia and seizure phenotypes. Rescuing any or all of these phenotypes by removing one of the cathepsins

would implicate that cathepsin in the pathogenesis of EPM1. We demonstrated that the severity of granule cell apoptosis, ataxia and seizures was not diminished by removal of cathepsins L or S, an indication that these cathepsins alone do not contribute to the symptoms of EPM1. However, when cathepsin B was eliminated from cystatin B-deficient mice, the number of cerebellar granule cells that died by apoptosis was greatly reduced, suggesting that cathepsin B initiates or propagates apoptosis when the inhibitor cystatin B is not present to curb these activities.

Because the removal of cathepsin B from cystatin B-deficient mice only partially reduced the apoptosis phenotype and did not rescue the ataxia and seizure phenotypes, we suspect that another cathepsin partially compensates for cathepsin B loss or that another factor contributes to the EPM1 pathogenesis. In support of this idea, studies by other investigators have demonstrated that removing cathepsin B from mice results in an incomplete rescue of cell death and resultant phenotypes (Guicciardi et al., 2000; Halangk et al., 2000; Guicciardi et al., 2001). Which other cathepsin could be compensating for or acting with cathepsin B in our system? Among the known cathepsins, we can probably exclude those with very low or undetectable expression in the brain (K, V, W, C, X) and the non-cysteine protease cathepsins that are not inhibited by cystatin B (D E, and G) as contributors to the phenotypes of EPM1. This leaves cathepsins L, S, H, F, and O. Cathepsin F is an attractive candidate because of its high expression levels in the brain (Wang, et al., 1998), and cathepsin H is interesting because it is relatively stable at neutral pH, a probable prerequisite for interacting with the cytoplasmic apoptotic pathway (reviewed in Turk et al., 2000). Although cathepsin L is one of the least stable cathepsins at neutral pH, it remains a good candidate because of its abundance in lysosomes, prevalence in the brain, and

reputed ability to cause apoptosis (Ishisaka et al., 1999). In fact, another study has suggested that cathepsin B and cathepsin L functionally compensate for each other *in vivo* because the phenotype of cathepsin B/L double knockout mice is considerably more severe than either of the single mutants alone (Felbor, et al., 2002). Studies are underway to address which other cysteine cathepsins or factors are responsible for the remaining EPM1 phenotypes.

Although lysosomes and their contents are not usually considered components of the apoptotic pathway, there is growing recognition that the traditional apoptotic pathways are unlikely to be the only ways to achieve apoptosis (Leist and Jaattela, 2001; Brunk et al., 2001; Turk et al., 2002). There is a great deal of experimental evidence supporting a role for cathepsins in apoptosis, and implicating cathepsin B in particular. Specifically, hepatocyte apoptosis induced by bile salts is known to involve cathepsin B (Roberts et al., 1997; Faubion et al., 1999), cathepsin B causes apoptosis in cultured primary granule cells (Kingham and Pocock, 2001), whereas inhibition of cathepsin B and L blocks apoptosis caused by p53 and various cytotoxic agents (Lotem and Sachs, 1996; Gray et al., 2001). Cathepsin B is relased from lysosomes by TNF-α signaling and is required for TNF-α dependent apoptosis in several systems (Monney et al., 1998; Guicciardi et al., 2000; Foghsgaard et al., 2001). Hepatocytes isolated from cathepsin B-deficient mice are greatly hindered in their ability to undergo TNF-α-mediated apoptosis (Guicciardi et al., 2001). In addition, the pro-apoptotic effects of active vitamin D have been ascribed to its ability to upregulate the expression of cathepsin B (Mathiasen et al., 2001). The case for cathepsin involvement in apoptosis initiation has been further strengthened by the recent finding that cathepsin L has been implicated in spermatogonial apoptosis (Fujimoto et al., 2002). In addition, the overexpression of cathepsin D induces apoptosis in cultured cells (Deiss et al.,

1996; Shibata et al., 1998) whereas cathepsin D inhibition or antisense treatment is protective (Deiss et al., 1996; Ishihara et al., 1999; Ollinger 2000; Zang et al., 2001).

Thus, it appears that cathepsins are capable of causing apoptosis, but what is the mechanism by which cathepsins enter into the apoptotic pathway? One possibility is that cathepsins cause generalized widespread proteolysis of cellular components, thereby inducing the cell to initiate apoptosis (Williams and Henkart, 1994). Alternatively, it may be that cathepsins directly cleave and thereby activate the traditional mediators of apoptosis, the caspases. It has been shown that cathepsin B can activate the inflammatory caspases 1 and 11 (Schotte, et al., 1998; Vancompernolle et al., 1998), serine cathepsin G can activate caspase 7 (Zhou and Salvesen, 1997), and cathepsin L may activate caspase 3 (Ishisaka et al. 1999). As another potential mechanism, cathepsins may translocate to the nucleus (Roberts et al., 1997) and cleave apoptotic effector substrates such as PARP (Biggs et al., 2001; Gobeil et al., 2001), although this does not yet appear to be a widespread phenomenon. A final mechanism was put forth by Stoka and colleagues, who determined that lysosomal extracts containing cathepsins could cleave the proapoptotic Bcl-2 family member Bid in a physiologically relevant manner that led to apoptosis (Stoka et al., 2000). In support of this finding, it was recently shown that selective disruption of lysosomes results in Bid activation and apoptosis (Reiners et al., 2002). In fact, cathepsins B, L, H, S, and K can cleave recombinant Bid in vitro, whereas cathepsins X and C can not (Boris Turk, unpublished data). Thus, it appears that cathepsins are capable of interfacing with the existing apoptotic machinery, but under what physiologic conditions do cathepsins actually become released from the lysosome and thereby allowed physical access to the cytoplasmic apoptotic machinery?

Lysosomes may leak their contents constitutively at low levels (Brunk and Ericsson, 1972), and lysosomal integrity can be lost in certain disease states and during normal aging (Nakamura, et al., 1989; Bi et al., 2000). It is also known that lysosomal membranes can become leaky as a result of oxidative stress (Ollinger and Brunk, 1995; Antunes et al., 2001; Ishisaka et al., 2001; Kagedal et al., 2001a), a likely consequence of seizures (Ueda, et al. 1997). Another way that lysosomal membranes become compromised *in vivo* involves TNF-mediated sphingosine production, which is capable of disrupting lysosomal membranes and allowing cathepsin-dependent apoptosis to occur (Kagedal et al., 2001b). Lysosomal rupture has even been implicated in p53-mediated apoptosis (Yuan et al., 2002). It is well known that acute lysosomal rupture typically leads to necrotic cell death, but only recently was it appreciated through a variety of pathologic and experimental conditions that moderate leakage from lysosomal membranes results in an apoptotic form of cell death (Sheen et al., 1994; Brunk, et al., 1997; Yuan et al., 1997; Ishisaka et al., 1998; Monney et al., 1998; Brunk and Svensson, 1999). This apoptotic death appears to be mediated by cathepsins, as active cathepsins were consistently shown to redistribute from lysosomes to the cytosol before apoptosis ensued (Roberg and Ollinger, 1998; Roberg, et al., 1999; Hishita et al., 2001; Roberg, 2001; Zang et al., 2001).

We believe that the apoptotic signals initiated or propagated by cathepsins may represent subtle but important aspects of the apoptotic response. Allowing lysosomal proteases to feed into the apoptotic pathway would be a useful way for the cell to sense moderate lysosomal damage or oxidative stress. Alternatively, cathepsins may represent a back-up apoptotic mechanism or a

way to amplify weak apoptotic signals when caspases are inhibited. It may be difficult to detect these less obtrusive forms of apoptosis given the more prominent contribution by caspases.

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Figure Legends

Figure 1: Granule cell apoptosis in cystatin B, cathepsin doubly-deficient mice. Tunel staining of cerebellar sections from 2 month old mice (a-c, g-i, m-o) or 8 month old mice (d-f, j-l, p-r) to visualize apoptosis. At two months of age there is a) no apoptosis in cystatin B +/+ cathepsin L +/+ wild type mice, whereas b) cystatin B -/- cathepsin L +/+ mice, and c) doubly-deficient cystatin B -/- cathepsin L -/- mice experience equivalent amounts of apoptotic granule cells. At eight months of age, there is d.) no apoptosis in cystatin B +/+ cathepsin L +/+ wild type mice, whereas e) cystatin B -/- cathepsin L +/+ mice, and f) doubly-deficient cystatin B -/- cathepsin L

-/- mice experience equivalent amounts of apoptotic granule cells. At two months of age there is g) no apoptosis in cystatin B +/+ cathepsin S +/+ wild type mice, whereas h) cystatin B -/- cathepsin S +/+ mice, and i) doubly-deficient cystatin B -/- cathepsin S -/- mice experience equivalent amounts of apoptotic granule cells. At eight months of age there is j) no apoptosis in cystatin B +/+ cathepsin S +/+ wild type mice, whereas k.) cystatin B -/- cathepsin S +/+ mice, and l) doubly-deficient cystatin B -/- cathepsin S -/- mice experience equivalent amounts of apoptotic granule cells. At two months of age there is m) no apoptosis in cystatin B +/+ cathepsin B +/+ wild type mice, n) cystatin B -/- cathepsin B +/+ mice experience high amounts of apoptosis, whereas o) doubly-deficient cystatin B -/- cathepsin B -/- mice have considerably fewer of apoptotic granule cells. At eight months of age there is p) no apoptosis in cystatin B +/+ cathepsin B +/+ wild type mice, whereas q) cystatin B -/- cathepsin B +/+ mice display moderate levels of apoptosis and r) doubly-deficient cystatin B -/- cathepsin B -/- mice have considerably fewer apoptotic granule cells. Scale bar = 200 μm (a-r).

Average number of apoptotic granule cells per field at 2, 4 and 8 months of age. Quantitation confirms that s) cystatin B -/- cathepsin L +/+ mice experience equivalent amounts of cell death as cystatin B -/- cathepsin L -/- mice at 2 and 8 months of age. Similarly, t) cystatin B -/- cathepsin S +/+ mice experience comparable amounts of cell death as cystatin B -/- cathepsin S -/- mice at 2, 4 and 8 months of age. u) cystatin B -/- cathepsin B -/- mice experience greatly reduced amounts of cell death compared with cystatin B -/- cathepsin B +/+ mice at 2, 4 and 8 months of age. Error bars = standard deviation.

Figure 2: Ataxia measurements in cystatin B, cathepsin doubly-deficient mice. The still (a, e) and rotating (b, f) rotorod were used to measure ataxia at 2, 4 and 8 months of age. The gait variability paradigm (c, d, g) was used to measure ataxia at 2, 4 and 8 months of age. When compared to cystatin B +/+ cathepsin L +/+ mice (thick line, diamonds), cystatin B -/- cathepsin L +/+ mice (dashed line, squares) show a slightly reduced ability to remain on the a) still or b) rotating rod by 8 months of age whereas cystatin B -/- cathepsin L -/- mice (thin line, triangles) show a greatly reduced ability to remain on the a) still or b) rotating rod by 8 months of age. c) cystatin B -/- cathepsin L +/+ mice (dashed line, squares) and cystatin B -/- cathepsin L -/- mice (thin line, triangles) both experience more gait variability than cystatin B +/+ cathepsin L +/+ mice (thick line, diamonds) at 8 months of age.

d) Cystatin B -/- cathepsin S +/+ mice (dashed line, squares) and cystatin B -/- cathepsin S -/- mice (thin line, triangles) both experience more gait variability than cystatin B +/+ cathepsin S +/+ mice (thick line, diamonds) at 8 months of age.

When compared to cystatin B +/+ cathepsin B +/+ mice (heavy line, diamonds), cystatin B -/- cathepsin B +/+ mice (small dashes, squares) show a reduced ability to remain on the e) still or f) rotating rod by 8 months of age whereas cystatin B -/- cathepsin B -/- mice (thin line, triangles) show a greatly reduced ability to remain on the e) still or f) rotating rod by 8 months of age. Note the additive effects of combining the inherently ataxic cystatin B+/+ cathepsin B-/- (large dashes, circles) with cystatin B -/- cathepsin B +/+ mice (small dashes, squares) to result in the worst performance cystatin B -/- cathepsin B -/- mice (thin line, triangles). g) cystatin B -/- cathepsin B +/+ mice (small dashes, squares), cystatin B -/- cathepsin B -/- mice (thin line, triangles) and cystatin B +/+ cathepsin B -/- mice (large dashes, circles) all experience more gait

variability than cystatin B +/+ cathepsin B +/+ mice (thick line, diamonds) at 8 months of age. Error bars = standard deviation.

Figure 3: Electrocorticographic recordings from cystatin B, cathepsin doubly-deficient mice show myoclonus and seizures. Representative examples of spontaneous bilateral cortical discharges associated with myoclonic jerks in a) cystatin B -/- cathepsin +/+ mouse, b) cystatin B -/- cathepsin L -/- mouse, c) cystatin B -/- cathepsin B -/- mouse. Bilateral electrographic seizure discharges from d) cystatin B -/- cathepsin +/+ mouse and e) cystatin B -/- cathepsin B -/- mouse. Calibration bars: a-c) 0.2 mv., 1 sec., d) 0.2 mv. 0.5 sec., e) 0.5 mv., 1 sec.

Table 1: Eye phenotype prevalence in eight-month-old cystatin B, cathepsin doubly-deficient mice.

GENOTYPE	Number of Mice with Phenotype/Total
Cystatin B -/- Cathepsin L +/+	2 /10 (20%)
Cystatin B -/- Cathepsin L -/-	5 / 9 (56%)
Cystatin B -/- Cathepsin S +/+	1 / 3 (33%)
Cystatin B -/- Cathepsin S -/-	0 / 4 (0%)
Cystatin B -/- Cathepsin B +/+	3 / 8 (38%)
Cystatin B -/- Cathepsin B -/-	1 / 7 (14%)